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(54) Polynucleotide and use thereof

(57) A first polynucleotide including at least two complementary regions that are complementary to a target nucleic acid and have a reverse configuration, a second polynucleotide complementary to the first polynucleotide, and uses thereof, are provided.

FIG. 1

Fig. 1A

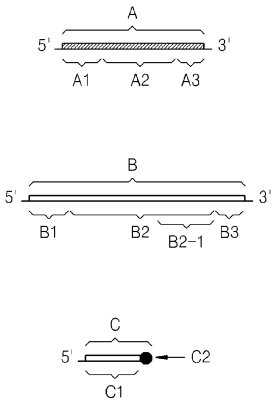
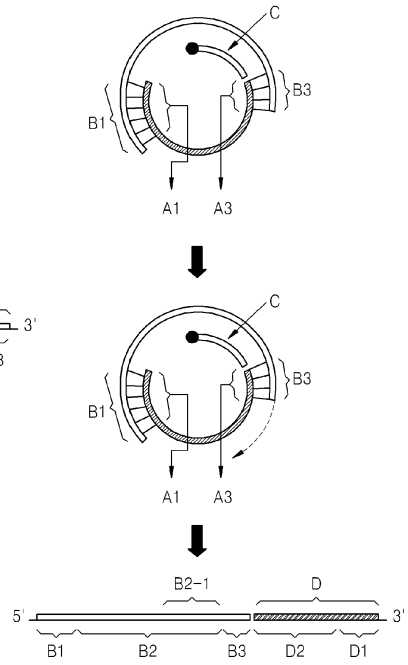


Fig. 1B



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Description

BACKGROUND

1. Field

[0001] The present disclosure relates to a first polynucleotide including at least two complementary regions that are complementary to a target nucleic, a second polynucleotide complementary to the first polynucleotide, and uses thereof.

2. Description of the Related Art

[0002] Methods of amplifying nucleic acids include extending a nucleotide sequence from the 3'-terminal of a primer in the presence of a nucleic acid polymerase. The primer includes a sequence complementary to that of a target nucleic acid. To extend nucleotide sequences, primers and target nucleic acids need to be specifically and stably hybridized with each other. The stability of the hybridized product of nucleic acids is known to be proportionate to the length of a complementary sequence. Meanwhile, if the length of a primer increases, the length of a target nucleic acid to be amplified shortens. Therefore, there is still a need to develop a polynucleotide that is specifically and stably binding to a target nucleic acid and increases the length of an amplified target nucleic acid.

BRIEF SUMMARY OF THE INVENTION

[0003] Provided are polynucleotides for efficiently amplifying target nucleic acids. The first polynucleotide comprises at least two complementary regions that are complementary to a target nucleic acid.

[0004] Provided are compositions and kits including the polynucleotides for efficiently amplifying target nucleic acids. The composition and kits comprise (a) a target nucleic acid; and (b) a first polynucleotide comprising at least two complementary regions that are complementary to the target nucleic acid. Provided are methods of efficiently producing a nucleotide sequence complementary to a target nucleic acid. The methods comprise (a) hybridizing a target nucleic acid with a first polynucleotide comprising at least two complementary regions that are complementary to a target nucleic acid to form a hybridized product and (b) incubating the hybridized product in the presence of a nucleic acid polymerase to extend a nucleotide sequence complementary to the target nucleic acid from the 3'-terminus of the first polynucleotide.

[0005] In the polypeptides, compositions, kits, and methods, the first complementary region of the first polypeptide is located at the 3'-terminal side of the polynucleotide and comprises at least one nucleotide that is complementary to at least one nucleotide of the 3'-terminal part of the target nucleic acid. The at least one second complementary region of the first polypeptide is located at the 5'-terminal side of the first complementary region and comprises at least one nucleotide which is complementary to at least one nucleotide of the 5'-terminal part of the target nucleic acid.

DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

[0006] These and/or other aspects will become apparent and more readily appreciated from the following description of the embodiments, taken in conjunction with the accompanying drawings of which:

[0007] FIG. 1A is a schematic diagram of a first polynucleotide (B), a second polynucleotide (C) and a target nucleic acid (A).

[0008] FIG. 1B is a schematic diagram showing a method of preparing a nucleotide sequence complementary to the target nucleic acid by using the first polynucleotide and the second polynucleotide.

[0009] FIG. 2 is a graph showing an effect of the length of a first complementary region (x-axis) on extension efficiency (Cp, y-axis), according to an embodiment of the present invention.

[0010] FIG. 3 is a graph showing effects of a reaction temperature (°C; x-axis) and a primer concentration on extension efficiency (Cp; y-axis) of the 3'-terminal of a reverse transcription primer (RTP) by reverse transcription, according to an embodiment of the present invention.

[0011] FIG. 4 is a graph showing the effect of the length of a second complementary region (nucleotides (nt); x-axis) on extension efficiency (Cp; y-axis) when miR-3141 is used as a target nucleic acid, according to an embodiment of the present invention.

[0012] FIG. 5 is a graph showing the effect of the length of a second complementary region (nucleotides (nt); x-axis) on extension efficiency (Cp; y-axis) when miR-16 is used as a target nucleic acid, according to an embodiment of the present invention.

[0013] FIG. 6 is a graph showing the effect of a first complementary region having a nucleotide sequence mismatched with a sequence of a target nucleic acid, according to an embodiment of the present invention. The position of the mismatch is indicated on the x-axis and the extension efficiency (Cp) is indicated on the y-axis.

[0014] FIG. 7 is a graph showing the effect of a first complementary region having a nucleotide sequence mismatched with a sequence of a target nucleic acid, according to an embodiment of the present invention. The position of the mismatch is indicated on the x-axis and the extension efficiency (Cp) is indicated on the y-axis.

[0015] FIG. 8 is a graph showing the effect of a first complementary region having a nucleotide sequence mismatched with a sequence of a target nucleic acid, according to an embodiment of the present invention. The position of the mismatch is indicated on the x-axis and the extension efficiency (Cp) is indicated on the y-axis.

[0016] FIGS. 9A and 9B are graphs illustrating the effect of a clamp and a clamp length on the efficiency of a reverse transcription. The RT primer is indicated on the x-axis and the extension efficiency (Cp) is indicated on the y-axis.

[0017] FIGS. 10A through 10E are graphs illustrating the effect of a clamp on the efficiency of a reverse transcription when the target nucleic acid is (A) miR- 16, (B) miR- 21, (C) miR210, (D) miR- 122- 5p, and (E) miR- 200c.

DETAILED DESCRIPTION OF THE INVENTION

[0018] Reference will now be made in detail to embodiments, examples of which are illustrated in the accompanying drawings, wherein like reference numerals refer to like elements throughout. In this regard, the present embodiments may have different forms and should not be construed as being limited to the descriptions set forth herein. Accordingly, the embodiments are merely described below, by referring to the figures, to explain aspects of the present description. As used herein, the term "and/or" includes any and all combinations of one or more of the associated listed items. Expressions such as "at least one of," when preceding a list of elements, modify the entire list of elements and do not modify the individual elements of the list.

[0019] According to an embodiment of the present invention, there is a first polynucleotide comprising at least two regions that are complementary to a target nucleic acid (herein referred to as "complementary regions"). The first complementary region of the at least two complementary regions is located at the 3'-terminal side of the first polynucleotide and comprises at least one nucleotide that is complementary to at least one nucleotide from the 3'-terminal part of the target nucleic acid. The at least one second complementary region of the at least two complementary regions is located at the 5'-terminal side of the first complementary region and comprises at least one nucleotide which is complementary to at least one nucleotide from the 5' terminal part of the target nucleic acid.

[0020] The first complementary region of the first polynucleotide may be complementary to at least one nucleotide from the 3'-terminal part of the target nucleic acid, including the 3'-terminal nucleotide of the target nucleic acid. For example, the first complementary region may be complementary to at least two consecutive nucleotides from the 3'-terminal part of the target nucleic acid, including the 3'-terminal nucleotide of the target nucleic acid. For example, the first complementary region may have consecutive nucleotides complementary to 2 to 50, 2 to 40, 2 to 30, 2 to 20, 2 to 10, 3 to 50, 4 to 40, 3 to 30, 3 to 20, 3 to 10, or 3 to 16 (e.g., 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 or 16) consecutive nucleotides of the target nucleic acid, including the 3'-terminal nucleotide of the target nucleic acid.

[0021] The second complementary region of the polynucleotide may be complementary to at least one nucleotide from the 5'-terminal part of the target nucleic acid. For example, the second complementary region may be complementary to at least one nucleotide from the 5'-terminal part of the target nucleic acid, including the 5'-terminal nucleotide of the target nucleic acid. For example, the at least one consecutive nucleotide may be 1 nucleotide (hereinafter, referred to as "nt") to 50 nt, for example, 1 nt to 40 nt, 1 nt to 30 nt, 1 nt to 20 nt, 1 nt to 10 nt, 2 nt to 40 nt, 2 nt to 30 nt, 2 nt to 20 nt, 2 nt to 10 nt, 3 nt to 40 nt, 3 nt to 30 nt, 3 nt to 20 nt, or 3 nt to 10 nt (e.g., 3 nt, 4 nt, 5 nt, 6 nt, 7 nt, 8 nt, 9 nt or 10 nt).

[0022] In this regard, the second complementary region may be complementary to at least two consecutive nucleotides of the target nucleic acid. For example, the second complementary region may have consecutive nucleotides complementary to 2 to 50, 2 to 40, 2 to 30, 2 to 20, 2 to 10, 3 to 50, 4 to 40, 3 to 30, 3 to 20, 3 to 10, or 3 to 16 (e.g., 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 or 16) consecutive nucleotides of the target nucleic acid, including the 5'-terminal nucleotide of the target nucleic acid.

[0023] In one embodiment, at least three consecutive nucleotides of the second complementary region may be complementary to the target nucleic acid. For example, the at least three consecutive nucleotides may be 3 nt to 50 nt, 3 nt to 40 nt, 3 nt to 30 nt, 3 nt to 20 nt, 3 nt to 10 nt, or 4 nt to 16 nt.

[0024] The first polynucleotide may include 1 to 3 (for example, 1, 2, or 3) second complementary region(s).

[0025] In the first polynucleotide, the first complementary region and the second complementary region may be separated from each other by at least one nucleotide. In one embodiment, the first complementary region and the second complementary region may be separated from each other by a linker. The linker may be, for example, a nucleic acid, a nucleic acid variant, protein, carbohydrate, lipid molecules, or a combination thereof. The nucleic acid variant may be peptide nucleic acid (PNA), locked nucleic acid (LNA), or a combination thereof. For example, the linker may include a nucleic acid having 0 to 50 nt. The length of the linker may be, for example, 1 nt to 50 nt, 2 nt to 50 nt, 2 nt to 40 nt, 2 nt to 30 nt, 2 nt to 20 nt, 2 nt to 10 nt, 3 nt to 50 nt, 4 nt to 40 nt, 3 nt to 30 nt, 3 nt to 20 nt, 3 nt to 10 nt, or 4 nt to 10 nt (e.g., 5 nt, 6 nt, 7 nt, 8 nt, or 9 nt). In one embodiment, the linker may be a primer binding site, a restriction enzyme recognition site, a probe binding site, or a combination thereof.

[0026] The first polynucleotide may be DNA or RNA. Also, examples of the polynucleotide include nucleotide analogues, for example, PNA and LNA. For example, the second complementary region of the polynucleotide may include a nucleotide analogue, for example, PNA, LNA, or a combination thereof. The nucleotide analogue, for example, PNA, LNA, or a combination thereof, may also be included in the first complementary region. The polynucleotide may be single-stranded. The length of the polynucleotide may be 7 nt to 200 nt, 7 nt to 180 nt, 7 nt to 150 nt, 7 nt to 130 nt, 7 nt to 100 nt, 7 nt to 80 nt, 7 nt to 50 nt, 7 nt to 30 nt, 7 nt to 20 nt, 7 nt to 15 nt, or 10 nt to 40 nt (e.g., 15 nt, 20 nt, 25 nt, 30 nt, or 35 nt).

[0027] The target nucleic acid may be DNA, RNA, or a chimera of DNA and RNA. The target nucleic acid may be single-stranded or double-stranded. The length of the target nucleic acid may be 20 nt to 200 nt, 20 nt to 180 nt, 20 nt to 150 nt, 20 nt to 130 nt, 20 nt to 100 nt, 20 nt to 80 nt, 20 nt to 50 nt, 20 nt to 30 nt, or 20 nt to 40 nt. The target nucleic acid may be small RNA (e.g., natural small RNA). For example, the small RNA may be microRNA, siRNA, tRNA, or a combination thereof. In addition, the target nucleic acid, which may be RNA having at least 200 nucleotides, may be RNA having a region where a sequence of 30 consecutive nucleotides has a GC content of less than 30% (e.g., less than 25%, less than 20%, less than 15%, less than 10%, or less than 5%) or of at least 80% (e.g., at least 85%, at least 90%, or at least 95%), RNA including at least 5 consecutive nucleotides having complementary sequences in molecules so as to form an intramolecular secondary structure, RNA including at least 5 consecutive nucleotides that are complementary to each other, or a combination thereof.

[0028] The first and second complementary regions of the polynucleotide may be hybridized to the target nucleic acid, being spaced apart from each other by at least 1 nt. For example, the first and second complementary regions may be spaced apart from each other by 1 nt to 20 nt, 1 nt to 10 nt, 1 nt to 5 nt, 2 nt, 3 nt, 4 nt, 5 nt, 6 nt, 7 nt, 8 nt, 9 nt, 10 nt, or 11 nt.

[0029] According to another embodiment of the present invention, there is provided a second polynucleotide including a third complementary region complementary to a region in the first polynucleotide. The third complementary region may be complementary to at least one nucleotide, preferably two or more consecutive nucleotides (e.g. 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30 nt), in the first polynucleotide, wherein said nucleotide(s) are separated from the first complementary region of the first polynucleotide.

[0030] The third complementary region may be at least one (consecutive) nucleotide starting from a location separated from the first complementary region of the first polynucleotide by about 0 to about 20 nt. For example, the third complementary region may be complementary to at least one nucleotide starting from a location spaced apart by 0 nt, 1 nt, 2 nt, 3 nt, 4 nt, 5 nt, 6 nt, 7 nt, 8 nt, 9 nt, 10 nt, 11 to 15 nt or 11 to 20 nt from the first complementary region of the first polynucleotide.

[0031] The second polynucleotide may have a length of 2 to 100 nt. For example, the second polynucleotide may have a length of 2 to 80 nt, 2 to 60, 2 to 40, 2 to 20 nt, 4 to 18 nt, 6 to 16 nt, or 8 to 14 nt, including 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 etc. nt.

[0032] The second polynucleotide may include DNA, RNA, PNA, LNA, nucleotide analogues or a combination thereof. The third complementary region of the second polynucleotide may include DNA, RNA, PNA, LNA, nucleotide analogues or a combination thereof. Also, the second polynucleotide may be single stranded.

[0033] The 3'-terminus of the second polynucleotide may be modified. The 3'-terminus of the second polynucleotide may, for example, include an inverted nucleotide, dideoxynucleotide, amine group, alkyl chain moiety or a combination thereof. The modified 3'-terminus may prevent the target nucleic acid from acting as a primer.

[0034] The first and second polynucleotides have multiple uses. For example, the first polynucleotide may act as a primer in template-dependent nucleic acid synthesis. Thus, the first polynucleotide may be used as a primer. The first polynucleotide may also be used as a probe for confirming a presence of the target nucleic acid in a sample. The second polynucleotide may be used as a clamp in a template-dependent nucleic acid synthesis to improve efficiency and specificity of a reverse transcription.

[0035] According to another embodiment of the present invention, there is provided a composition and kit for amplifying a target nucleic acid. The composition and kit include a target nucleic acid, and a first polynucleotide including at least two complementary regions that are complementary to the target nucleic acid.

[0036] The composition and kit may further include the second polynucleotide including a third complementary region complementary to part of the first polynucleotide. The third complementary region may be complementary to at least one (consecutive) nucleotide(s) separated from the first complementary region of the first polynucleotide.

[0037] The composition and kit may be used for amplifying the target nucleic acid. The amplification process may be performed using one of various known methods of amplifying nucleic acids. The amplification of the target nucleic acid may be DNA amplification or RNA amplification. The amplification process may be performed under thermal cycling or isothermal conditions. Examples of the amplification method include a polymerase chain reaction (PCR), nucleic acid sequence-based amplification (NASBA), a ligase chain reaction (LCR), strand displacement amplification (SDA), rolling circle amplification (RCA), and the like. Also, the amplification method may include a method of amplifying RNA. For example, the amplification method may include reverse transcription (RT) or RT-PCR. The amplification process may be a process of increasing the copy number of target nucleic acid sequences or sequences complementary thereto. The term "PCR" used herein refers to a method of amplifying a target nucleic acid from primer pairs specifically binding to

the target nucleic acid by using a polymerase.

[0038] Thus, the composition and kit may further include a material known for the amplification of the target nucleic acid. For example, the composition and kit may further include a nucleic acid polymerase, a buffer for the activity of the nucleic acid polymerase, a cofactor, and/or a matrix. The nucleic acid polymerase may be one selected from a DNA polymerase, a RNA polymerase, a reverse transcriptase, and a combination thereof. The term "reverse transcription" may refer to the synthesis of DNA strands that are complementary to RNA sequences by using RNA as a template. The nucleic acid polymerase may have strand displacement activity. For example, the nucleic acid polymerase may be at least one reverse transcriptase derived from retrovirus, for example, HIV (Human Immunodeficiency Virus), MMLV (Moloney Murine Leukemia Virus), or AMV (Avian Myeloblastosis Virus) . The nucleic acid polymerase may not have 3'- > 5' exonuclease activity. The composition and kit may include a material for reverse transcription or PCR amplification. The composition and kit may further include an instruction manual used to amplify the target nucleic acid.

[0039] According to another embodiment of the present invention, there is provided a method of producing a nucleotide sequence complementary to a target nucleic acid, the method including (a) hybridizing the target nucleic acid with a first polynucleotide including at least two complementary regions that are complementary to the target nucleic acid to form a hybridized product and (b) incubating the hybridized product in the presence of a nucleic acid polymerase to extend a nucleotide sequence complementary to the target nucleic acid from the 3'-terminus of the first polynucleotide.

[0040] The hybridization process may be performed using one of various known methods. For example, the hybridization process may be performed by incubating the first polynucleotide and the target nucleic acid in a known buffer appropriate for the hybridization of nucleic acids. The hybridization process may be performed at a temperature ranging from about 0 °C to about 25 °C, for example, 4 °C. The hybridization temperature may be appropriately adjusted according to the sequences and lengths of selected first polynucleotide and target nucleic acid. The hybridization process may be performed for an appropriate time period, for example, about 1 to about 12 hours (overnight).

[0041] The method includes incubating the hybridized product in the presence of a nucleic acid polymerase to extend a nucleotide sequence complementary to the target nucleic acid from the 3'-terminus of the polynucleotide.

[0042] The incubating process may be performed under conditions appropriate for the activity of the nucleic acid polymerase. The incubating process may be performed in the presence of the nucleic acid polymerase, a buffer for the activity of the nucleic acid polymerase, a cofactor, and a substrate for the enzymes. The nucleic acid polymerase may be one selected from a DNA polymerase, a RNA polymerase, a reverse transcriptase, and a combination thereof. The term "reverse transcription" may refer to the synthesis of DNA strands that are complementary to RNA sequences by using RNA as a template. The nucleic acid polymerase may have strand displacement activity. For example, the nucleic acid polymerase may be a reverse transcriptase derived from retrovirus, for example, HIV, MMLV, or AMV. The nucleic acid polymerase may not have 3'-> 5' exonuclease activity. For example, the incubating process may be performed in the presence of a material for RT or PCR amplification.

[0043] Via the incubating process, the nucleotide sequence complementary to the target nucleic acid may be extended from the 3'-terminus of the first polynucleotide. The extending process includes extending from the 3'-terminus of the first polynucleotide, with the nucleic acid polymerase displacing the 5'-terminal part of the first polynucleotide hybridized with the target nucleic acid, see Fig. 1B.

[0044] The method may further include hybridizing a hybridized product and a second polynucleotide. The second polynucleotide may be a polynucleotide including a third complementary region complementary to part of the first polynucleotide. The third complementary region may be complementary to at least one (consecutive) nucleotide separated from the first complementary region of the first polynucleotide.

[0045] Regarding the method, hybridizing the first polynucleotide and the target nucleic acid, and hybridizing the hybridized product and the second polynucleotide may be performed simultaneously or consecutively. For example, the target nucleic acid and the first polynucleotide may be hybridized and then the hybridized product and the second polynucleotide may be hybridized; the first polynucleotide and the second polynucleotide may be hybridized and then the hybridized product and the target nucleic acid may be hybridized; or the first polynucleotide, the second polynucleotide, and the target nucleic acid may be hybridized simultaneously.

[0046] The method may further include determining whether or not the produced product, i.e., the nucleotide sequence complementary to the target nucleic acid, is present. The method may further include determining that, as a result of the above-described determination, if the produced product is present, the target nucleic acid exists in a sample, and on the other hand, if the produced product is not present, the target nucleic acid does not exist in the sample.

[0047] In addition, the method may further include amplifying a nucleic acid by using as a template the produced product, i.e., the nucleotide sequence complementary to the target nucleic acid. The amplification process may be performed using one of various known methods as described herein.

[0048] The method may further include determining whether or not the amplified product, i.e., the nucleotide sequence complementary to the target nucleic acid, is present. The method may further include determining that, as a result of the above-described determination process, if the amplified product is present, the target nucleic acid exists in a sample, and on the other hand, if the amplified product is not present, the target nucleic acid does not exist in the sample.

[0049] The method may include hybridizing a target nucleic acid with a polynucleotide including at least two complementary regions that are complementary to the target nucleic acid, wherein a first complementary region of the at least two complementary regions is located at the 3'-terminal side of the polynucleotide and comprises at least one nucleotide mismatched with a nucleotide of the target nucleic acid and at least one nucleotide which is complementary to a nucleotide from the 3'-terminal part of the target nucleic acid, and at least one second complementary region of the at least two complementary regions which is located at the 5'-terminal side of the first complementary region and comprises at least one nucleotide which is complementary to a nucleotide from the 5'-terminal part of the target nucleic acid.

[0050] The first complementary region may include 1 to 4 nt mismatches, for example, 1, 2, 3, or 4 nt mismatch(es). The mismatch may be one nucleotide mismatch located at a 1st, 2nd, 3rd, 4th , 5th, or 6th position from the 3'-terminal of the first complementary region. The method may further include comparing a product extended by using the polynucleotide containing the mismatch with a product extended by using the polynucleotide not including the mismatch.

[0051] In addition, the method may further include determining whether or not a mutation is present in the target nucleic acid based on the comparison results. For example, the method may further include determining that if a product extended from the polynucleotide including the mismatch is obtained, but a product extended from the polynucleotide not including the mismatch is not obtained, a nucleotide of the target nucleic acid that corresponds to the mismatch has a mutation. Also, the method may further include determining that if a product extended from the polynucleotide including the mismatch is not obtained, but a product extended from the polynucleotide not including the mismatch is obtained, a nucleotide of the target nucleic acid that corresponds to the mismatch has no mutation.

[0052] FIGS. 1A and B illustrate a method of preparing a nucleotide sequence complementary to a target nucleic acid using a first polynucleotide and a second polynucleotide, according to an embodiment of the present invention. As shown in FIGS. 1A and B, the target nucleic acid A includes a 5'-terminal region A1, a middle region A2, and a 3'-terminal region A3. The first polynucleotide B includes a first complementary region B3 which is complementary to the 3'-terminal part A3 of the target nucleic acid, a region B2 which is non-complementary to the target nucleic acid, and a second complementary region B1 which is complementary to the 5'-terminal part A1 of the target nucleic acid. The region non-complementary to the target nucleic acid B2 includes a region B2-1 which is complementary to the third complementary region C1 of the second polynucleotide C. The second polynucleotide C includes a third complementary region C1, and the 3'-terminus thereof may be a modified nucleotide C2. The third complementary region C1 of the second polynucleotide may be separated from the first polynucleotide by at least one nucleotide at a 3'-terminal of a region non-complementary B2 to the target nucleic acid. A hybridization of the first polynucleotide, the second polynucleotide and the target nucleic acid facilitates an efficient reverse transcription by a reverse transcriptase because the third complementary region C1 of the second polynucleotide is arranged in the 3'-terminal direction from the target nucleic acid A. When the reverse transcription is completed, a cDNA D is produced, including cDNA D1 at the 5'-terminal region A1 of the target nucleic acid, and cDNA D2 in the middle region A2 of the target nucleic acid.

[0053] Since the first complementary region (B3) and the second complementary region (B1) are complementary to the target nucleic acid (A), they synergically contribute to the hybridization, and thus may synergically contribute to the stabilization of the hybridized product. Therefore, the length of the first complementary region, which contributes to the priming of nucleic acid amplification, may be shortened without loss of specificity, sensitivity, and/or a speed of amplification. In addition, when the 3'-terminus of the polynucleotide is extended by a nucleic acid polymerase (refer to D of FIG. 1), the target nucleic acid may be amplified. In FIG. 1B, the extension of the 3'-terminal of the polynucleotide includes reverse transcription or synthesis of DNA strands from DNA strands. The first and second complementary regions hybridized with the target nucleic acid may be spaced apart from each other by at least 1 nt. Also, the second polynucleotide may be decreased a length of the first complementary region used for priming, such that a length of the reverse transcribed product may be lengthened, PCR primer design may be easier, and the specificity of the reverse transcription may be increased.

EXAMPLES

[0054] One or more embodiments of the present invention will now be described more detailed with reference to the following examples. However, these examples are provided only for illustrative purposes and are not intended to limit the scope of the invention.

Example 1. Preparation of reverse transcription primer and clamp

[0055] A sequence complementary to a target nucleic acid was extended from the 3'-terminal of a first polynucleotide (hereinafter, referred to as "reverse transcription primer," "RT primer," or "RTP") including at least two complementary regions that are complementary to a target nucleic acid, wherein a first complementary region of the at least two complementary regions is located at the 3'-terminal side of the first polynucleotide and comprises at least one nucleotide complementary to the 3'-terminal part of the target nucleic acid, and wherein at least one second complementary region

of the at least two complementary regions is located at the 5'-terminal side of the first complementary region and includes at least one nucleotide which is complementary to the 5'-terminal part of the target nucleic acid. As a control, a general primer having no reverse configuration (hereinafter, referred to as "linear primer) was used. The "reverse transcription primer" may be reciprocally exchanged with the "reverse configuration primer." The first complementary region of the RTP is referred to as a "priming region," and the second complementary region of the RTP is referred to as an "adaptor region" or an "adaptor."

[0056] Also, a second polynucleotide (hereinafter, "clamp" or "clamp nucleotide") including a third complementary region complementary to part of the RTP was prepared, wherein the clamp has the third complementary region complementary to one or more consecutive nucleotides separated from the first complementary region of the RTP.

Example 2. Effect of Length of First Complementary Region on Extension Efficiency

[0057] An extension reaction of the RTP was performed by varying the length of the first complementary region from a 3mer to a 7mer. Reverse transcription was performed as the extension reaction.

[0058] Superscript III™ (Invitrogen) was used as a reverse transcriptase. The reverse transcription was performed by incubating the RTP and a RNA template in 50 mM Tris-HCl (pH 8.3 at room temperature) containing 37.5 mM KCl, 3 mM$MgCl_2$, and 10 mM DTT at 42 °C for 1 hour. As the RNA template, miR-16 RNA having a sequence of SEQ ID NO: 9 with a 20 nt tag sequence added to the 5'-terminal thereof was used. The Superscript III™ reverse transcriptase was a variant of Moloney murine leukemia virus (M-MLV) reverse transcriptase manipulated to have decreased RNA H activity and increased thermal stability.

[0059] The extended product was amplified by PCR. The PCR was performed by adding 50 nM of each of a primer pair and the extended product to a 2xSYBR RT-PCR master mixture (Exiqon) and thermally cycling the resulting mixture. The thermally cycling process was performed under the following conditions: 95 °C for 10 minutes, and 45 cycles, each cycle at 95 °C for 15 seconds, and at 60°C for 1 minute. Next, melting curve analysis was performed by 5 measurement/ °C. RT primers used were RTP-3mer (SEQ ID NO: 1), RTP-4mer (SEQ ID NO: 2), RTP-6mer (SEQ ID NO: 3), RTP-7mer (SEQ ID NO: 4), RTP-3mer (SEQ ID NO: 5), Linear-4mer (SEQ ID NO: 6), Linear-6mer (SEQ ID NO: 7), and Linear-7mer (SEQ ID NO: 8). The PCR primers used were a forward primer (SEQ ID NO: 10) and a reverse primer (SEQ ID NO: 11).

[0060] FIG. 2 is a graph showing an effect of the length of a first complementary region on extension efficiency. As illustrated in FIG. 2, when the length of the first complementary region is a 3mer and 4mer, the RTP had a significantly decreased cross-point (Cp) value as compared to the linear primer correlating with increased extension efficiency. When the length of the first complementary region is a 4mer, the Cp value of the RTP was 6.3 smaller than that of the control. Cp value represents the cycle by which the fluorescence of a sample increased to a level higher than the background fluorescence in the amplification cycle, and also represents the cycle in which a second derivative of amplification curve has the maximum value. The Cp value was decreased because the sample was amplified in less number of cycles, and thus extension efficiency was increased.

Example 3. Effects of Reaction Temperature and Primer Concentration on Extension Efficiency

[0061] The target nucleic acid was amplified using the RTP having a length of a 6mer having a little difference in extension efficiency from that of the control. The RTP and the control had sequences of SEQ ID NOS: 12 and 13, respectively, and a target RNA (miR-3141 RNA with a 20 nt tag sequence added to the 5'-terminal thereof) had a sequence of SEQ ID NO: 14. The concentrations of the RTP used were 1 nM, 10 nM, and 100 nM, respectively, and reverse transcription temperatures used were 42 °C, 50 °C, and 55 °C, respectively. The reverse transcription and PCR conditions except for the conditions listed above were the same as those described in Example 1 above.

[0062] FIG. 3 is a graph showing effects of a reaction temperature and a primer concentration on extension efficiency of the 3'-terminal of the RTP by reverse transcription. As illustrated in FIG. 3, the RTP had decreased Cp values as compared to the control.

Example 4. Effect of Length of Second Complementary Region on Extension Efficiency

[0063] The effect of the length of the second complementary region on extension efficiency of the RTP in transcription and PCR amplification was confirmed.

[0064] As target RNAs, miR-16 with a 20 nt tag sequence added to the 5'-terminal thereof (SEQ ID NO: 9) and miR-3141 with a 20 nt tag sequence added to the 5'-terminal thereof (SEQ ID NO: 14) were used. The RTPs used with respect to miR-3141 were RTPs with second complementary regions having lengths of 4mer, 8mer, 10mer, 12mer, 14mer, and 15mer that are respectively added to the 5'-terminal thereof and complementary to the target RNA (SEQ ID NOS: 15, 16, 17, 18, 19, and 20, respectively). The RTPs used with respect to miR-16 were RTPs with second comple-

mentary regions having lengths of 4mer, 8mer, 10mer, 12mer, 14mer, and 16mer that are respectively added to the 5'-terminal thereof and complementary to the target RNA (SEQ ID NOS: 21, 22, 23, 24, 25, and 26, respectively).

[0065] FIG. 4 is a graph showing an effect of the length of a second complementary region on extension efficiency when miR-3141 is used as a target nucleic acid. As shown in FIG. 4, the RTP including the second complementary region having a length of 10mer had the lowest Cp value.

[0066] FIG. 5 is a graph showing an effect of the length of a second complementary region on extension efficiency when miR-16 is used as a target nucleic acid. As shown in FIG. 5, the RTP including the second complementary region having a length of 12mer had the lowest Cp value.

[0067] Table 1 (miR-3141) and Table 2 (miR-16) show characteristics of the second complementary region.

<Table 1 >

SEQ ID No. of RTP	Length of second complementary region in RTP	Sequence of second complementary region in RTP	GC content (%)	basic Tm (°C)	Salt concentration-adjusted Tm (°C)	NN Tm (°C)
SEQ ID No. 15	4	CCTC	3	14	14	-
SEQ ID No. 16	8	CCGCCCTC	7	30	30	18.73
SEQ ID No. 17	10	ACCCGCCCTC	8	36	36	31.49
SEQ ID No. 18	12	CCACCCGCCCTC	10	44	44	40.98
SEQ ID No. 19	14	CTCCACCCGCCCTC	11	49.1	52.7	46.58

<Table 2>

SEQ ID No. of RTP	Length of second complementary region in RTP	Sequence of second complementary region in RTP	GC content (%)	basic Tm (°C)	Salt concentration-adjusted Tm (°C)	NN Tm (°C)
SEQ ID No. 21	4	TGCT	2	12	12	-
SEQ ID No. 22	8	GTGCTGCT	5	26	26	8.98
SEQ ID No. 23	10	ACGTGCTGCT	6	32	32	28.32
SEQ ID No. 24	12	TTACGTGCTGCT	6	36	36	33.97
SEQ ID No. 25	14	ATTTACGTGCTGCT	6	34.4	38	38.41
SEQ ID No. 26	16	ATATTTACGTGCTGC T	6	38.3	43.2	41.29

[0068] In Tables 1 and 2, NN denotes nearest neighbor.

Example 5. Effect of First Complementary Region Including Nucleotide Sequence Mismatched with Target Nucleic Acid on Extension Efficiency

[0069] An effect of a RTP including a first complementary region having a nucleotide sequence mismatched with a

target nucleic acid sequence on extension efficiency in transcription and PCR amplification was confirmed.

[0070] A primer having no mismatch sequence and having a sequence complementary to the target nucleic acid (hereinafter, referred to as "wild-type (WT)") and primers having mismatch sequences that correspond to the 1st, 2nd, 3rd 4th 5th, and 6th positions, respectively, from the 3'-terminal of miR-3141 (M1, M2, M3, M4, M5, and M6, respectively) were used. In this regard, first complementary regions having the lengths of 0mer, 1mer, 2mer, 3mer, 4mer, and 6mer were used. When the length of the first complementary region is a 6mer, the WT primer and the M1, M2, M3, M4, M5 and M6 primers have sequences of SEQ ID NOS: 27, 28, 29, 30, 31, 32, and 33, respectively. As the target nucleic acid, miR-3141 RNA having a sequence of SEQ ID NO: 14 was used.

[0071] When the length of the first complementary region is a 4mer, the WT primer and the M1, M2, M3 and M4 primers have sequences of SEQ ID NOS: 34, 35, 36, 37, and 38, respectively.

[0072] When the length of the first complementary region is a 3mer, the WT primer and the M1, M2 and M3 primers have sequences of SEQ ID NOS: 39, 40, 41, and 42, respectively.

[0073] When the length of the first complementary region is a 2mer, the WT primer and the M1 and M2 primers have sequences of SEQ ID NOS: 43, 44, and 45, respectively.

[0074] When the length of the first complementary region is a 1 mer, the WT primer and the M1 primer have sequences of SEQ ID NOS: 46 and 47, respectively.

[0075] When the length of the first complementary region is a 0mer, the WT primer has a sequence of SEQ ID NO: 48.

[0076] The concentrations of each primer used were 10 nM, 100 nM, and 1 μM, respectively. The reverse transcription and PCR conditions, except for the condition described above, are the same as those described in Example 1 above.

[0077] FIGS. 6 through 8 are graphs showing an effect of the first complementary region including a nucleotide sequence mismatched with a sequence of a target nucleic acid on extension efficiency. The concentrations of each primer illustrated in FIGS. 6 through 8 were 1 μM, 100 nM, and 10 nM, respectively. As shown in FIGS. 6 through 8, Cp values had a significant difference between the case where the first complementary region of the RTP had the mismatch sequence and the case where the first complementary region of the RTP did not have the mismatch sequence. Referring to FIGS. 6 through 8, when the length of the first complementary region is a 4mer, the Cp values of the mismatch primers were 6.8 to 10.7 greater than that of the WT primer. In FIGS. 6 through 8, M1, M2, M3, M4, M5, and M6 denote mismatch positions from the 3'- terminal of miR- 3141 having a sequence of cggugagguc uuugguucau gagggcgggu ggag (M6) g (M5) a (M4) g (M3) g (M2) a (M1) (SEQ ID NO: 14) .

[0078] The results of FIGS. 6 through 8 indicate that it may be confirmed whether or not mutation is present in the target nucleic acid by introducing a mismatch sequence to the first complementary region. Whether or not mutation is present in the target nucleic acid may be confirmed by comparing a product amplified from the mismatch primer with a product amplified from the primer having no mismatch sequence.

Example 6. Effect of Clamp and Clamp Length on Efficiency of Reverse Transcription

[0079] A reverse transcription reaction was performed by including a clamp or changing a clamp length to about a 6mer to about a 16mer.

[0080] Superscript III™ (Invitrogen) was used as a reverse transcriptase. The reverse transcriptase and a RNA template was incubated for an hour in 50 mM Tris-HCl, (pH 8.3 at a room temperature) containing 37.5 mM KCl, 3 mM $MgCl_2$, and 10 mM DTT, for an hour at 42 °C. miR-16 (SEQ ID NO: 49) was used as the RNA template. Superscript III™ is an M-MLV (Moloney murine leukemia virus) reverse transcriptase variant engineered to reduce RNase H activity and to provide increased thermostability.

[0081] The resulting reverse transcription products were amplified by PCR. The PCR was performed by adding 50 nM of each primer, and reverse transcription products to a 2x SYBR RT-PCR master mixture (Exiqon) and thermal cycling thereof. The thermal cycling is performed 45 times, a cycling consisting of 10 minutes at 95 °C, 15 seconds at 95 °C, and 1 minute at 60 °C. Subsequently, melting curve analysis was performed by 5 measurement/°C. Table 3 illustrates the sequences of the used template, a PCR primer, a reverse transcription primer, and a clamp. 'A0' as used in Table 3 refers to a linear primer.

<Table 3>

	Name	Sequence
Template	miR16	5'-UAGCAGCACGUAAAUAUUGGCG-3' (SEQ ID NO: 49)
PCR primer	Forward primer	5'-CGCGCTAGCAGCACGTAAAT-3' (SEQ ID NO: 50)
	Reverse primer	5'-GTGCAGGGTCCGAGGT-3' (SEQ ID NO: 51)

(continued)

	Name	Sequence
RT primer	A12 RT0	5'-TACGTGCTGCTA GTGCAGGGTCCGAGGT ATTCGCACTGGATACGAC-3' (SEQ ID NO: 52)
	A12 RT1	5'-TACGTGCTGCTA GTGCAGGGTCCGAGGT ATTCGCACTGGATACGAC C-3' (SEQ ID NO: 53)
	A12 RT2	5'-TACGTGCTGCTA GTGCAGGGTCCGAGGT ATTCGCACTGGATACGAC CG-3' (SEQ ID NO: 54)
	A12 RT3	5'-TACGTGCTGCTA GTGCAGGGTCCGAGGT ATTCGCACTGGATACGAC CGC-3'(SEQ ID NO: 55)
	A12 RT4	5'-TACGTGCTGCTA GTGCAGGGTCCGAGGT ATTCGCACTGGATACGAC CGCC-3'(SEQ ID NO: 56)
	A12 RT6	5'-TACGTGCTGCTA GTGCAGGGTCCGAGGT ATTCGCACTGGATACGAC CGCCAA-3'(SEQ ID NO: 57)
	A0 RT0	5'-GTGCAGGGTCCGAGGT ATTCGCACTGGATACGAC-3' (SEQ ID NO: 58)
	A0 RT1	5'-GTGCAGGGTCCGAGGT ATTCGCACTGGATACGAC C-3' (SEQ ID NO: 59)
	A0 RT2	5'-GTGCAGGGTCCGAGGT ATTCGCACTGGATACGAC CG-3' (SEQ ID NO: 60)
	A0 RT3	5'-GTGCAGGGTCCGAGGT ATTCGCACTGGATACGAC CGC-3' (SEQ ID NO: 61)
	A0 RT4	5'-GTGCAGGGTCCGAGGT ATTCGCACTGGATACGAC CGCC-3' (SEQ ID NO: 62)
	A0 RT6	5'-GTGCAGGGTCCGAGGT ATTCGCACTGGATACGAC CGCCAA-3' (SEQ ID NO: 63)
Clamp	C16	5'-GTCGTATCCAGTGCGA-3' (SEQ ID NO: 64)
	C14	5'-GTCGTATCCAGTGC-3' (SEQ ID NO: 65)
	C12	5'-GTCGTATCCAGT-3' (SEQ ID NO: 66)
	C10	5'-GTCGTATCCA-3' (SEQ ID NO: 67)
	C8	5'-GTCGTATC-3' (SEQ ID NO: 68)
	C6	5'-GTCGTA-3' (SEQ ID NO: 69)

[0082] FIG. 9A illustrates an effect of a clamp on a reverse transcription efficiency, and FIG. 9B illustrates an effect of a clamp length on a reverse transcription efficiency. As shown in FIG. 9A, Cp value is reduced when the reverse transcription primer is used instead of the linear primer, and when the clamp is used, the Cp value decreased significantly. As shown in FIG. 9B, the Cp value decreased as the clamp length increased. During the reverse transcription reaction, a length of a 3'-terminal priming region may be reduced according to a clamp usage.

Example 7. Effect of a 3' Priming Region of Reverse Transcription Primer Length and Clamp on Reverse Transcription Efficiency

[0083] A reverse transcription was performed by decreasing a 3'-terminal region length of a reverse transcription primer.

[0084] Reaction compositions and conditions for the reverse transcription and PCR are shown in Example 2.

[0085] Tables 4 through 8 illustrate the sequences of used templates (miR16, miR21, miR122-5p, and miR200c, respectively), PCR primers, and reverse transcription primers. Clamp C10 (SEQ ID NO: 67) in Example 2 was used as a clamp.

<Table 4>

	Name	Sequence
Template	miR16	5'-UAGCAGCACGUAAAUAUUGGCG-3' (SEQ ID NO: 49)

(continued)

	Name	Sequence
PCR primer	Forward primer	5'-CGCGCTAGCAGCACGTAAAT-3' (SEQ ID NO: 50)
	Reverse primer	5'-GTGCAGGGTCCGAGGT-3' (SEQ ID NO: 51)
RT primer	V1-RTO	5'-TACGTGCTGCTA GTGCAGGGTCCGAGGT ACTGGATACGAC-3' (SEQ ID NO: 70)
	V1-RT1	5'-TACGTGCTGCTA GTGCAGGGTCCGAGGT ACTGGATACGAC C-3' (SEQ ID NO: 71)
	V1-RT2	5'-TACGTGCTGCTA GTGCAGGGTCCGAGGT ACTGGATACGAC CG-3' (SEQ ID NO: 72)
	V1-RT3	5'-TACGTGCTGCTA GTGCAGGGTCCGAGGT ACTGGATACGAC CGC-3' (SEQ ID NO: 73)
	V1-RT4	5'-TACGTGCTGCTA GTGCAGGGTCCGAGGT ACTGGATACGAC CGCC-3' (SEQ ID NO: 74)
	V1-RT5	5'-TACGTGCTGCTA GTGCAGGGTCCGAGGT ACTGGATACGAC CGCCA-3' (SEQ ID NO: 75)
	V1-RT6	5'-TACGTGCTGCTA GTGCAGGGTCCGAGGT ACTGGATACGAC CGCCAA-3' (SEQ ID NO: 76)
	V1-RT7	5'-TACGTGCTGCTA GTGCAGGGTCCGAGGT ACTGGATACGAC CGCCAAT-3' (SEQ ID NO: 77)
	V1-RT8	5'-TACGTGCTGCTA GTGCAGGGTCCGAGGT ACTGGATACGAC CGCCAATA-3' (SEQ ID NO: 78)
	Linear-RT0	5'-GTGCAGGGTCCGAGGT-3' (SEQ ID NO: 79)
	Linear -RT1	5'-GTGCAGGGTCCGAGGT C-3' (SEQ ID NO: 80)
	Linear -RT2	5'-GTGCAGGGTCCGAGGT CG-3' (SEQ ID NO: 81)
	Linear -RT3	5'-GTGCAGGGTCCGAGGT CGC-3' (SEQ ID NO: 82)
	Linear -RT4	5'-GTGCAGGGTCCGAGGT CGCC-3' (SEQ ID NO: 83)
	Linear -RT5	5'-GTGCAGGGTCCGAGGT CGCCA-3' (SEQ ID NO: 84)
	Linear -RT6	5'-GTGCAGGGTCCGAGGT CGCCAA-3' (SEQ ID NO: 85)
	Linear -RT7	5'-GTGCAGGGTCCGAGGT CGCCAAT-3' (SEQ ID NO: 86)
	Linear -RT8	5'-GTGCAGGGTCCGAGGT CGCCAATA-3' (SEQ ID NO: 87)

<Table 5>

	Name	Sequence
Template	miR21	5'-uagcuuaucagacugauguuga-3' (SEQ ID NO: 88)
PCR primer	Forward primer	5'-cgg tagcttatcagactgatgt-3' (SEQ ID NO: 89)
	Reverse primer	5'-GTGCAGGGTCCGAGGT-3' (SEQ ID NO: 90)
RT primer	V1-RTO	5'-TCTGATAAGCTA GTGCAGGGTCCGAGGT ACTGGATACGAC-3' (SEQ ID NO: 91)
	V1-RT1	5'-TCTGATAAGCTA GTGCAGGGTCCGAGGT ACTGGATACGAC T-3' (SEQ ID NO: 92)
	V1-RT2	5'-TCTGATAAGCTA GTGCAGGGTCCGAGGT ACTGGATACGAC TC-3' (SEQ ID NO: 93)

(continued)

	Name	Sequence
	V1-RT3	5'-TCTGATAAGCTA GTGCAGGGTCCGAGGT ACTGGATACGAC TCA-3' (SEQ ID NO: 94)
	V1-RT4	5'-TCTGATAAGCTA GTGCAGGGTCCGAGGT ACTGGATACGAC TCAA-3' (SEQ ID NO: 95)
	V1-RT5	5'-TCTGATAAGCTA GTGCAGGGTCCGAGGT ACTGGATACGAC TCAAC-3' (SEQ ID NO: 96)
	V1-RT6	5'-TCTGATAAGCTA GTGCAGGGTCCGAGGT ACTGGATACGAC TCAACA-3' (SEQ ID NO: 97)
	V1-RT7	5'-TCTGATAAGCTA GTGCAGGGTCCGAGGT ACTGGATACGAC TCAACAT-3' (SEQ ID NO: 98)
	V1-RT8	5'-TCTGATAAGCTA GTGCAGGGTCCGAGGT ACTGGATACGAC TCAACATC-3' (SEQ ID NO: 99)
	Linear -RT0	5'-GTGCAGGGTCCGAGGT-3' (SEQ ID NO: 100)
	Linear -RT1	5'-GTGCAGGGTCCGAGGT T-3' (SEQ ID NO: 101)
	Linear -RT2	5'-GTGCAGGGTCCGAGGT TC-3' (SEQ ID NO: 102)
	Linear -RT3	5'-GTGCAGGGTCCGAGGT TCA-3' (SEQ ID NO: 103)
	Linear -RT4	5'-GTGCAGGGTCCGAGGT TCAA-3' (SEQ ID NO: 104)
	Linear -RT5	5'-GTGCAGGGTCCGAGGT TCAAC-3' (SEQ ID NO: 105)
	Linear -RT6	5'-GTGCAGGGTCCGAGGT TCAACA-3' (SEQ ID NO: 106)
	Linear -RT7	5'-GTGCAGGGTCCGAGGT TCAACAT-3' (SEQ ID NO: 107)
	Linear -RT8	5'-GTGCAGGGTCCGAGGT TCAACATC-3' (SEQ ID NO: 108)

<Table 6>

	Name	Sequence
Template	miR210	5'-CUGUGCGUGUGACAGCGGCUGA-3' (SEQ ID NO: 109)
PCR primer	Forward primer	5'-CTGTGCGTGTGACAGC-3' (SEQ ID NO: 110)
	Reverse primer	5'-GTGCAGGGTCCGAGGT-3' (SEQ ID NO: 111)
RT primer	V1-RTO	5'-TCACACGCACAG GTGCAGGGTCCGAGGT ACTGGATACGAC-3' (SEQ ID NO: 112)
	V1-RT1	5'-TCACACGCACAG GTGCAGGGTCCGAGGT ACTGGATACGAC T-3' (SEQ ID NO: 113)
	V1-RT2	5'-TCACACGCACAG GTGCAGGGTCCGAGGT ACTGGATACGAC TC-3' (SEQ ID NO: 114)
	V1-RT3	5'-TCACACGCACAG GTGCAGGGTCCGAGGT ACTGGATACGAC TCA-3' (SEQ ID NO: 115)
	V1-RT4	5'-TCACACGCACAG GTGCAGGGTCCGAGGT ACTGGATACGAC TCAG-3' (SEQ ID NO: 116)
	V1-RT5	5'-TCACACGCACAG GTGCAGGGTCCGAGGT ACTGGATACGAC TCAGC-3' (SEQ ID NO: 117)
	V1-RT6	5'-TCACACGCACAG GTGCAGGGTCCGAGGT ACTGGATACGAC TCAGCC-3' (SEQ ID NO: 118)

(continued)

	Name	Sequence
	V1-RT7	5'-TCACACGCACAG GTGCAGGGTCCGAGGT ACTGGATACGAC TCAGCCG-3' (SEQ ID NO: 119)
	V1-RT8	5'-TCACACGCACAG GTGCAGGGTCCGAGGT ACTGGATACGAC TCAGCCGC-3' (SEQ ID NO: 120)
	Linear-RT0	5'-GTGCAGGGTCCGAGGT-3' (SEQ ID NO: 121)
	Linear-RT1	5'-GTGCAGGGTCCGAGGT T-3' (SEQ ID NO: 122)
	Linear-RT2	5'-GTGCAGGGTCCGAGGT TC-3' (SEQ ID NO: 123)
	Linear-RT3	5'-GTGCAGGGTCCGAGGT TCA-3' (SEQ ID NO: 124)
	Linear-RT4	5'-GTGCAGGGTCCGAGGT TCAG-3' (SEQ ID NO: 125)
	Linear-RT5	5'-GTGCAGGGTCCGAGGT TCAGC-3' (SEQ ID NO: 126)
	Linear-RT6	5'-GTGCAGGGTCCGAGGT TCAGCC-3' (SEQ ID NO: 127)
	Linear-RT7	5'-GTGCAGGGTCCGAGGT TCAGCCG-3' (SEQ ID NO: 128)
	Linear-RT8	5'-GTGCAGGGTCCGAGGT TCAGCCGC-3' (SEQ ID NO: 129)

<Table 7>

	Name	Sequence
Template	miR122-5p	5'-UGGAGUGUGACAAUGGUGUUUG-3' (SEQ ID NO: 130)
PCR primer	Forward primer	5'-CGTGGAGTGTGACAATGG-3' (SEQ ID NO: 131)
	Reverse primer	5'-GTGCAGGGTCCGAGGT-3' (SEQ ID NO: 132)
RT primer	V1-RTO	5'-TGTCACACTCCA GTGCAGGGTCCGAGGT ACTGGATACGAC-3' (SEQ ID NO: 133)
	V1-RT1	5'-TGTCACACTCCA GTGCAGGGTCCGAGGT ACTGGATACGAC C-3' (SEQ ID NO: 134)
	V1-RT2	5'-TGTCACACTCCA GTGCAGGGTCCGAGGT ACTGGATACGAC CA-3' (SEQ ID NO: 135)
	V1-RT3	5'-TGTCACACTCCA GTGCAGGGTCCGAGGT ACTGGATACGAC CAA-3' (SEQ ID NO: 136)
	V1-RT4	5'-TGTCACACTCCA GTGCAGGGTCCGAGGT ACTGGATACGAC CAAA-3' (SEQ ID NO: 137)
	V1-RT5	5'-TGTCACACTCCA GTGCAGGGTCCGAGGT ACTGGATACGAC CAAAC-3' (SEQ ID NO: 138)
	V1-RT6	5'-TGTCACACTCCA GTGCAGGGTCCGAGGT ACTGGATACGAC CAAACA-3' (SEQ ID NO: 139)
	V1-RT7	5'-TGTCACACTCCA GTGCAGGGTCCGAGGT ACTGGATACGAC CAAACAC-3' (SEQ ID NO: 140)
	V1-RT8	5'-TGTCACACTCCA GTGCAGGGTCCGAGGT ACTGGATACGAC CAAACACC-3' (SEQ ID NO: 141)
	Linear -RT0	5'-GTGCAGGGTCCGAGGT-3' (SEQ ID NO: 142)
	Linear -RT1	5'-GTGCAGGGTCCGAGGT C-3' (SEQ ID NO: 143)
	Linear -RT2	5'-GTGCAGGGTCCGAGGT CA-3' (SEQ ID NO: 144)
	Linear -RT3	5'-GTGCAGGGTCCGAGGT CAA-3' (SEQ ID NO: 145)

(continued)

	Name	Sequence
	Linear -RT4	5'-GTGCAGGGTCCGAGGT CAAA-3' (SEQ ID NO: 146)
	Linear -RT5	5'-GTGCAGGGTCCGAGGT CAAAC-3' (SEQ ID NO: 147)
	Linear -RT6	5'-GTGCAGGGTCCGAGGT CAAACA-3' (SEQ ID NO: 148)
	Linear -RT7	5'-GTGCAGGGTCCGAGGT CAAACAC-3' (SEQ ID NO: 149)
	Linear -RT8	5'-GTGCAGGGTCCGAGGT CAAACACC-3' (SEQ ID NO: 150)

<Table 8>

	Name	Sequence
Template	miR200c	5'-UAAUACUGCCGGGUAAUGAUGGA-3' (SEQ ID NO: 151)
PCR primer	Forward primer	5'-GGTAATACTGCCGGGTAATGA-3' (SEQ ID NO: 152)
	Reverse primer	5'-GTGCAGGGTCCGAGGT-3' (SEQ ID NO: 153)
RT primer	V1-RTO	5'-CCGGCAGTATTA GTGCAGGGTCCGAGGT ACTGGATACGAC-3' (SEQ ID NO: 154)
	V1-RT1	5'-CCGGCAGTATTA GTGCAGGGTCCGAGGT ACTGGATACGAC T-3' (SEQ ID NO: 155)
	V1-RT2	5'-CCGGCAGTATTA GTGCAGGGTCCGAGGT ACTGGATACGAC TC-3' (SEQ ID NO: 156)
	V1-RT3	5'-CCGGCAGTATTA GTGCAGGGTCCGAGGT ACTGGATACGAC TCC-3' (SEQ ID NO: 157)
	V1-RT4	5'-CCGGCAGTATTA GTGCAGGGTCCGAGGT ACTGGATACGAC TCCA-3' (SEQ ID NO: 158)
	V1-RT5	5'-CCGGCAGTATTA GTGCAGGGTCCGAGGT ACTGGATACGAC TCCAT-3' (SEQ ID NO: 159)
	V1-RT6	5'-CCGGCAGTATTA GTGCAGGGTCCGAGGT ACTGGATACGAC TCCATC-3' (SEQ ID NO: 160)
	V1-RT7	5'-CCGGCAGTATTA GTGCAGGGTCCGAGGT ACTGGATACGAC TCCATCA-3' (SEQ ID NO: 161)
	V1-RT8	5'-CCGGCAGTATTA GTGCAGGGTCCGAGGT ACTGGATACGAC TCCATCAT-3' (SEQ ID NO: 162)
	Linear -RT0	5'-GTGCAGGGTCCGAGGT-3' (SEQ ID NO: 163)
	Linear -RT1	5'-GTGCAGGGTCCGAGGT T-3' (SEQ ID NO: 164)
	Linear -RT2	5'-GTGCAGGGTCCGAGGT TC-3' (SEQ ID NO: 165)
	Linear -RT3	5'-GTGCAGGGTCCGAGGT TCC-3' (SEQ ID NO: 166)
	Linear -RT4	5'-GTGCAGGGTCCGAGGT TCCA-3' (SEQ ID NO: 167)
	Linear -RT5	5'-GTGCAGGGTCCGAGGT TCCAT-3' (SEQ ID NO: 168)
	Linear -RT6	5'-GTGCAGGGTCCGAGGT TCCATC-3' (SEQ ID NO: 169)
	Linear -RT7	5'-GTGCAGGGTCCGAGGT TCCATCA-3' (SEQ ID NO: 170)
	Linear -RT8	5'-GTGCAGGGTCCGAGGT TCCATCAT-3' (SEQ ID NO: 171)

[0086] FIGS. 10A through 10E illustrate an effect of decreasing a 3- terminal priming region length on efficiency of a reverse transcription corresponding to a clamp (FIG. 10A: miR- 16, FIG. 10B: miR- 21, FIG. 10C: miR- 210, FIG. 10D:

miR- 122- 5p, FIG. 10E: miR- 200c) . As shown in FIGS. 10A through 10E, 3- terminal priming region length may be reduced by using a clamp during the reverse transcription reaction.

[0087] As described above, according to the one or more of the above embodiments of the present invention, a polynucleotide may be used to efficiently amplify a target nucleic acid. A composition and kit including the polynucleotide may be used to efficiently amplify the target nucleic acid. A method of producing a nucleotide sequence complementary to the target nucleic acid may be used to efficiently produce the nucleotide sequence complementary to the target nucleic acid.

[0088] It should be understood that the exemplary embodiments described therein should be considered in a descriptive sense only and not for purposes of limitation. Descriptions of features or aspects within each embodiment should typically be considered as available for other similar features or aspects in other embodiments.

[0089] The use of the terms "a" and "an" and "the" and "at least one" and similar referents in the context of describing the invention (especially in the context of the following claims) are to be construed to cover both the singular and the plural, unless otherwise indicated herein or clearly contradicted by context. The use of the term "at least one" followed by a list of one or more items (for example, "at least one of A and B") is to be construed to mean one item selected from the listed items (A or B) or any combination of two or more of the listed items (A and B), unless otherwise indicated herein or clearly contradicted by context. The terms "comprising," "having," "including," and "containing" are to be construed as open-ended terms (i.e., meaning "including, but not limited to,") unless otherwise noted. Recitation of ranges of values herein are merely intended to serve as a shorthand method of referring individually to each separate value falling within the range, unless otherwise indicated herein, and each separate value is incorporated into the specification as if it were individually recited herein. All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (e.g., "such as") provided herein, is intended merely to better illuminate the invention and does not pose a limitation on the scope of the invention unless otherwise claimed. No language in the specification should be construed as indicating any non-claimed element as essential to the practice of the invention.

[0090] Preferred embodiments of this invention are described herein, including the best mode known to the inventors for carrying out the invention. Variations of those preferred embodiments may become apparent to those of ordinary skill in the art upon reading the foregoing description. The inventors expect skilled artisans to employ such variations as appropriate, and the inventors intend for the invention to be practiced otherwise than as specifically described herein. Accordingly, this invention includes all modifications and equivalents of the subject matter recited in the claims appended hereto as permitted by applicable law. Moreover, any combination of the above-described elements in all possible variations thereof is encompassed by the invention unless otherwise indicated herein or otherwise clearly contradicted by context.

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<120> Polynucleotide and use thereof

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<210> 8
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aggtggtagc cttgaggaca cgatcgtcct cc 32

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cctcaggtgg tagccttgag gacatcct 28

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<223> RCP with 5′ terminal 8mer adaptor sequence

<400> 16
ccgccctcag gtggtagcct tgaggacatc ct 32

<210> 17
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acccgccctc aggtggtagc cttgaggaca tcct 34

<210> 18
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ccacccgccc tcaggtggta gccttgagga catcct 36

<210> 19
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ctccacccgc cctcaggtgg tagccttgag gacatcct 38

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cctccacccg ccctcaggtg gtagccttga ggacatcct 39

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tgctaggtgg tagccttgag gacacgcc 28

<210> 22
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<220>
<223> RCP with 5′ terminal 8mer adaptor sequence complementary to miR-16

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gtgctgctag gtggtagcct tgaggacacg cc 32

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<220>
<223> RCP with 5′ terminal 10mer adaptor sequence complementary to miR-16

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<210> 24
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<223> RCP with 5′ terminal 12mer adaptor sequence complementary to miR-16

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<220>
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<220>
<223> RCP with 5′ terminal 16mer adaptor sequence complementary to miR-16

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<210> 28
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<400> 30
acccgccctc aggtggtagc cttgaggaca tcatcc 36

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acccgccctc aggtggtagc cttgaggaca tccacc 36

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acccgccctc aggtggtagc cttgaggaca tcctca 36

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acccgccctc aggtggtagc cttgaggaca tcct 34

<210> 35
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<400> 35
acccgccctc aggtggtagc cttgaggaca acct 34

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<400> 36
acccgccctc aggtggtagc cttgaggaca tact 34

<210> 37
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acccgccctc aggtggtagc cttgaggaca tcat 34

<210> 38
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<210> 39

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acccgccctc aggtggtagc cttgaggaca tcc 33

<210> 40
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<400> 41
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<400> 42
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<210> 44

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<210> 45
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<220>
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<400> 45
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<210> 46
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acccgccctc aggtggtagc cttgaggaca a 31

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<223> Forward primer for miR-16

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cgcgctagca gcacgtaaat 20

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<400> 51
gtgcagggtc cgaggt 16

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tacgtgctgc tagtgcaggg tccgaggtat tcgcactgga tacgac 46

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<400> 65
gtcgtatcca gtgc 14

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<220>
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gtcgtatcca 10

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<400> 68
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gtcgta 6

<210> 70
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<400> 77
tacgtgctgc tagtgcaggg tccgaggtac tggatacgac cgccaat 47

<210> 78
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gtgcagggtc cgaggtc 17

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gtgcagggtc cgaggtcgc 19

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gtgcagggtc cgaggtcgcc aa 22

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gtgcagggtc cgaggtcgcc aat 23

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gtgcagggtc cgaggtcgcc aata 24

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uagcuuauca gacugauguu ga 22

<210> 89

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cggtagctta tcagactgat gt 22

<210> 90
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gtgcagggtc cgaggt 16

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ctgtgcgtgt gacagc 16

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gtgcagggtc cgaggt 16

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gtgcagggtc cgaggtt 17

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gtgcagggtc cgaggttc 18

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Claims

1. A polynucleotide comprising at least two complementary regions that are complementary to a target nucleic acid, wherein a first complementary region is located at the 3'-terminal side of the polynucleotide and comprises at least one nucleotide that is complementary to the 3'-terminal part of the target nucleic acid, and
wherein at least one second complementary region is located at the 5'-terminal side of the first complementary region and comprises at least one nucleotide that is complementary to the 5'-terminal part of the target nucleic acid.

2. The polynucleotide of claim 1, wherein the second complementary region comprises at least one nucleotide that is complementary to the 5'-terminal part of the target nucleic acid including the 5'-terminal nucleotide.

3. The polynucleotide of claim 1 or 2, wherein the first complementary region comprises at least one nucleotide that is complementary to the 3'-terminal part of the target nucleic acid including the 3'-terminal nucleotide.

4. The polynucleotide of claim 3, wherein the first complementary region comprises 2 to 7 consecutive nucleotides that are complementary to the 3'-terminal of the target nucleic acid including the 3'-terminal nucleotide.

5. The polynucleotide of any of claims 1 to 4, wherein the first complementary region and the second complementary region are spaced apart from each other by a linker.

6. A composition for amplifying a target nucleic acid, the composition comprising:

(a) a target nucleic acid; and
(b) a first polynucleotide comprising at least two complementary regions that are complementary to the target nucleic acid,
wherein a first complementary region is located at the 3'-terminal side of the polynucleotide and comprises at least one nucleotide that is complementary to the 3'-terminal part of the target nucleic acid, and
wherein at least one second complementary region is located at the 5'-terminal side of the first complementary region and comprises at least one nucleotide that is complementary to the 5'-terminal part of the target nucleic acid.

7. The composition of claim 6, further comprising a second polynucleotide, wherein the second polynucleotide comprises a third complementary region that is complementary to a part of the first polynucleotide.

8. The composition of claim 7, wherein the third complementary region is complementary to at least one nucleotide separated from the first complementary region of the first polynucleotide.

9. The composition of claim 7 or 8, wherein the third complementary region is complementary to at least one nucleotide separated from the first complementary region of the first polynucleotide by about 0 to about 20 nucleotides.

10. The composition of any of claims 7 to 9, wherein the 3'-terminus of the second polynucleotide is modified, preferably to comprise an inverted nucleotide, dideoxynucleotide, an amine group, an alkyl chain moiety.

11. A method of producing a nucleotide sequence complementary to a target nucleic acid, the method comprising:

(a) hybridizing a target nucleic acid with a first polynucleotide comprising at least two complementary regions that are complementary to a target nucleic acid to form a hybridized product,
wherein a first complementary region of the at least two complementary regions is located at the 3'-terminal side of the polynucleotide and comprises at least one nucleotide that is complementary to the 3'-terminal part of the target nucleic acid, and
wherein at least one second complementary region of the at least two complementary regions is located at the 5'-terminal side of the first complementary region and comprises at least one nucleotide that is complementary to the 5'-terminal part of the target nucleic acid; and
(b) incubating the hybridized product in the presence of a nucleic acid polymerase to extend a nucleotide sequence complementary to the target nucleic acid from the 3'-terminus of the first polynucleotide.

12. The method of claim 11, wherein the nucleic acid polymerase is a strand displacement nucleic acid polymerase, preferably a reverse transcriptase derived from HIV (Human Immunodeficiency Virus), MMLV (Moloney Murine Leukemia Virus), or AMV (Avian Myeloblastosis Virus).

13. The method of claim 11 or 12, wherein the target nucleic acid is RNA, preferably microRNA, siRNA, tRNA, non-coding RNA, or a combination thereof.

14. The method of any of claims 11 to 13, wherein the method further comprises hybridizing the hybridized product of (a) to a second polynucleotide, wherein the second polynucleotide comprises a third complementary region complementary to at least one nucleotide of the first polynucleotide, wherein the third complementary region is separated from the first complementary region.

15. The method of claim 14, wherein hybridizing the first polynucleotide and the target nucleic acid, and hybridizing the hybridized product to the second polynucleotide are performed simultaneously or consecutively.

FIG. 1

Fig. 1A

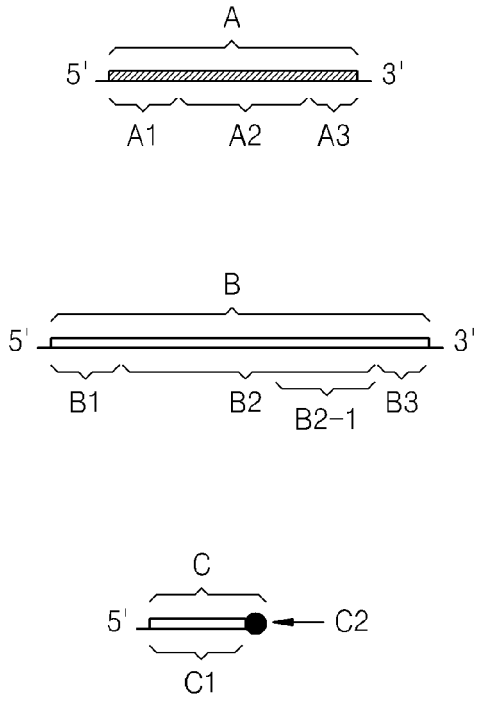


Fig. 1B

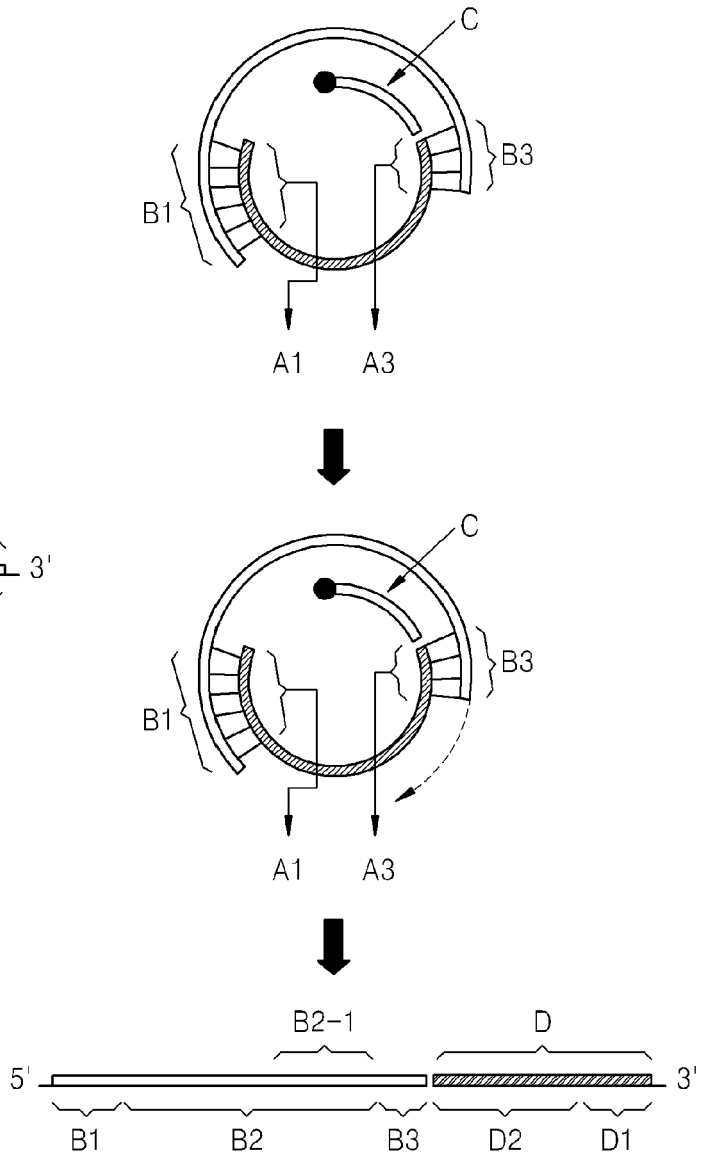


FIG. 2

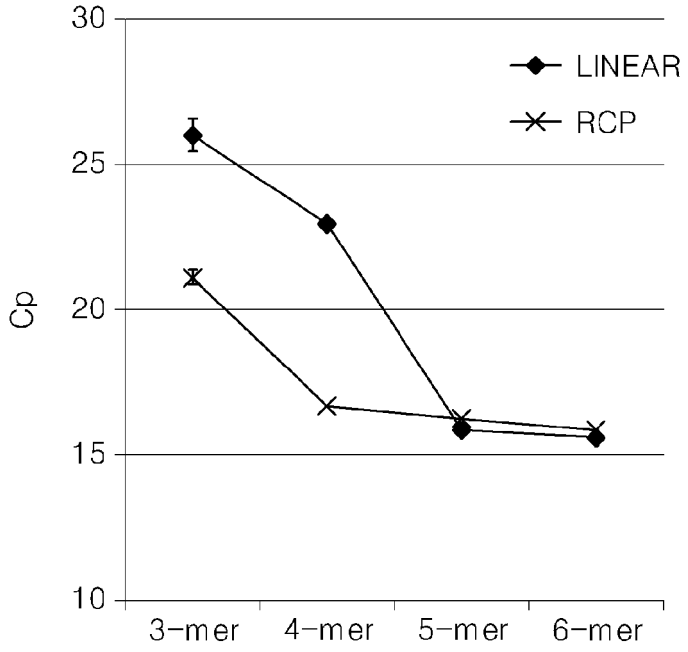

30
25
20
15
10
Cp
LINEAR
RCP
3-mer
4-mer
5-mer
6-mer
LENGTH OF FIRST COMPLEMENTARY REGION

FIG. 3

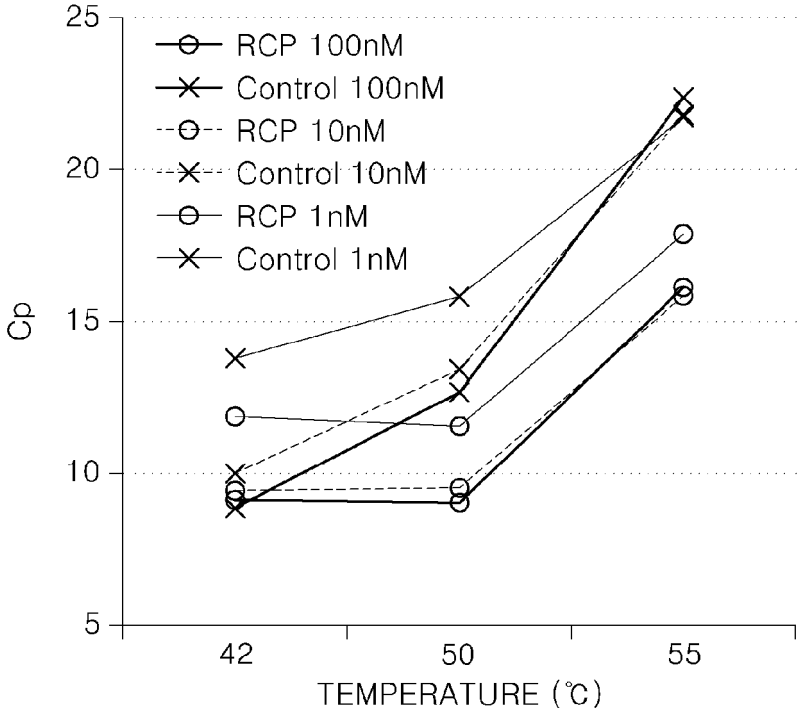

25
20
15
10
5
Cp
RCP 100nM
Control 100nM
RCP 10nM
Control 10nM
RCP 1nM
Control 1nM
42
50
55
TEMPERATURE (℃)

FIG. 4

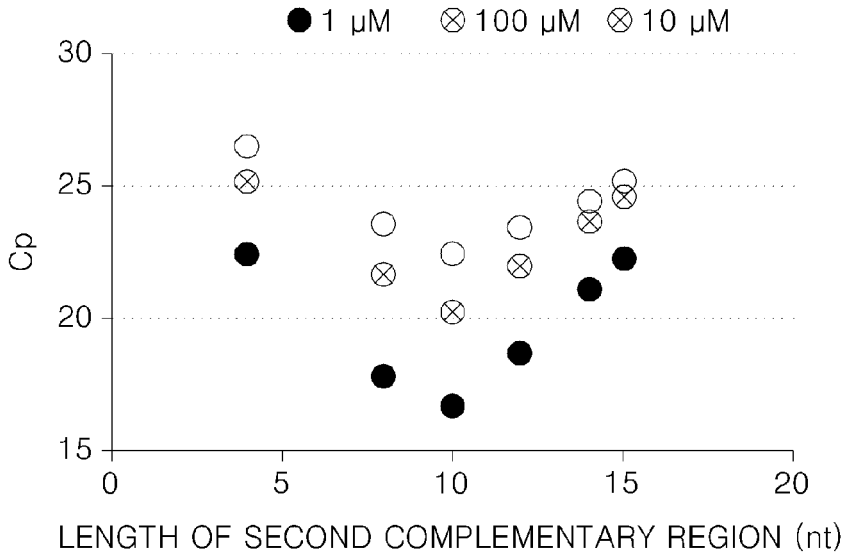

1 μM
100 μM
10 μM
30
25
20
15
Cp
0
5
10
15
20
LENGTH OF SECOND COMPLEMENTARY REGION (nt)

FIG. 5

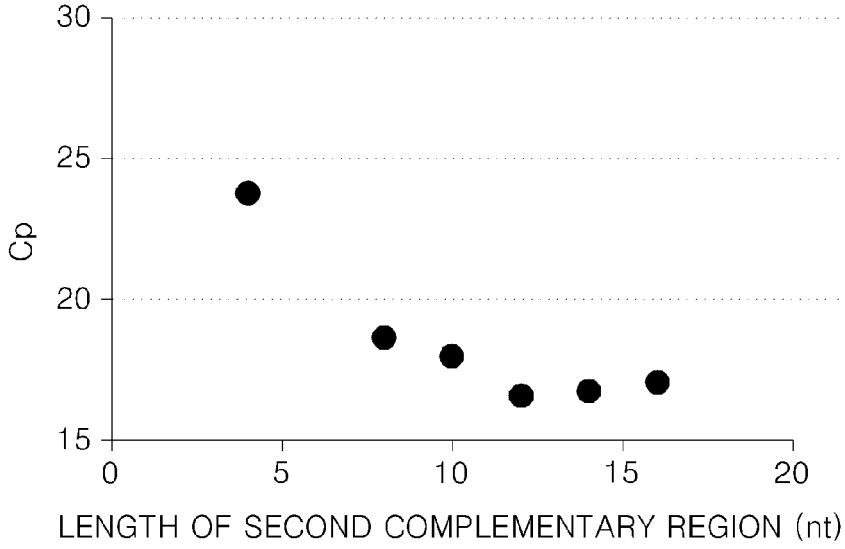

30
25
20
15
Cp
0
5
10
15
20
LENGTH OF SECOND COMPLEMENTARY REGION (nt)

FIG. 6

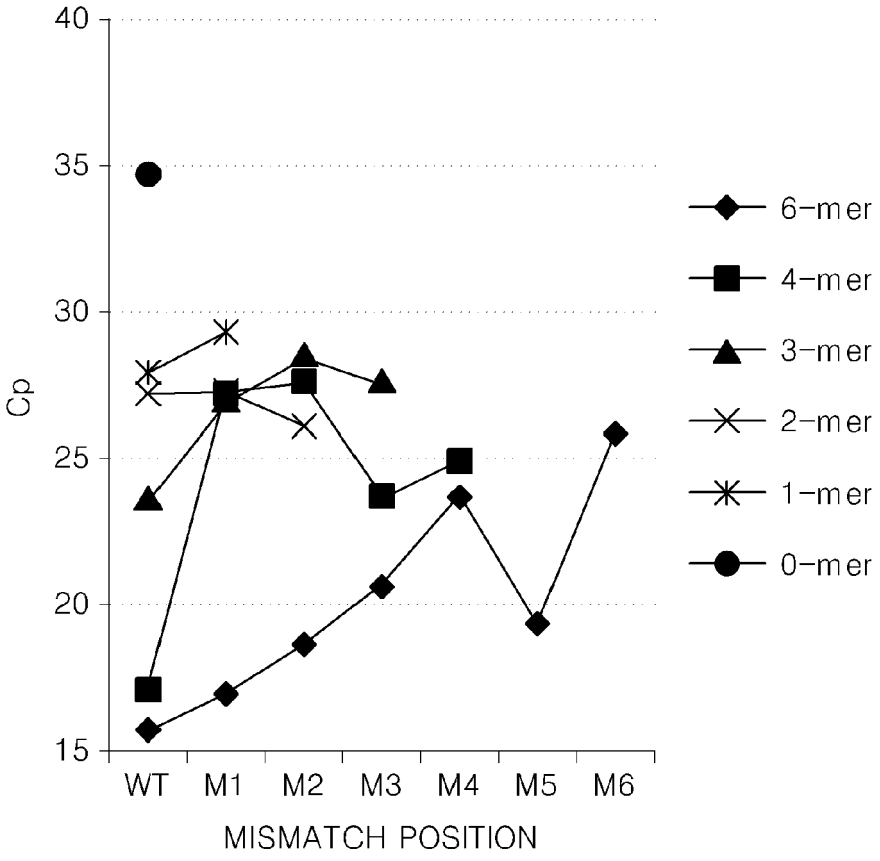

40
35
30
25
20
15
Cp
WT
M1
M2
M3
M4
M5
M6
MISMATCH POSITION
6-mer
4-mer
3-mer
2-mer
1-mer
0-mer

FIG. 7

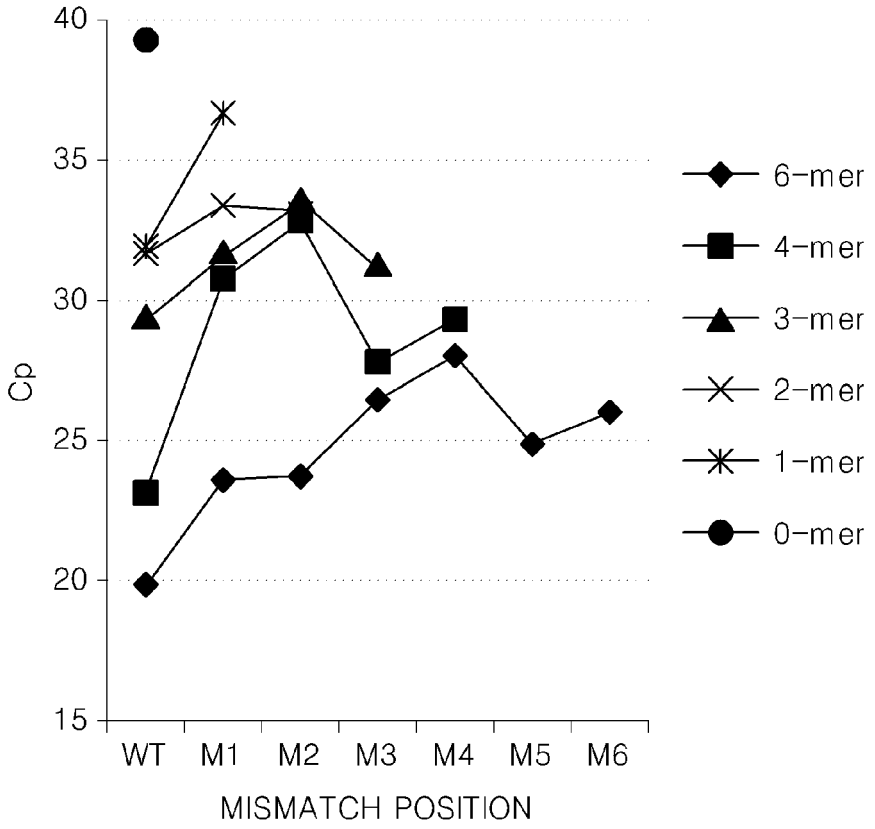

40
35
30
25
20
15
Cp
WT
M1
M2
M3
M4
M5
M6
MISMATCH POSITION
6-mer
4-mer
3-mer
2-mer
1-mer
0-mer

FIG. 8

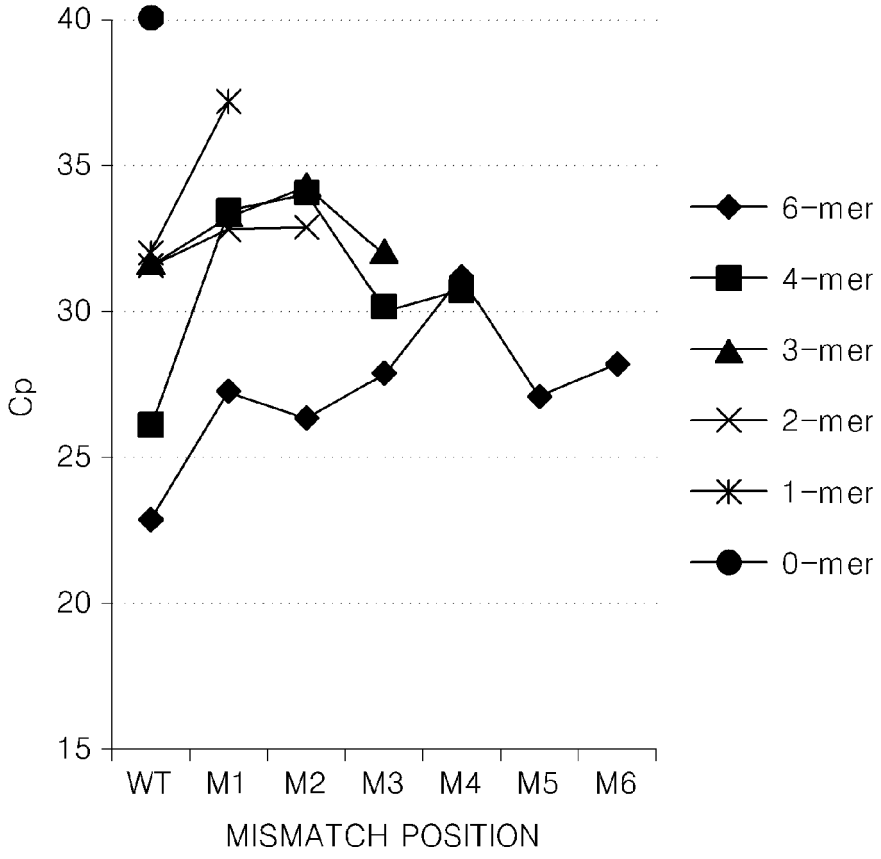

40
35
30
25
20
15
Cp
WT
M1
M2
M3
M4
M5
M6
MISMATCH POSITION
6-mer
4-mer
3-mer
2-mer
1-mer
0-mer

FIG. 9A

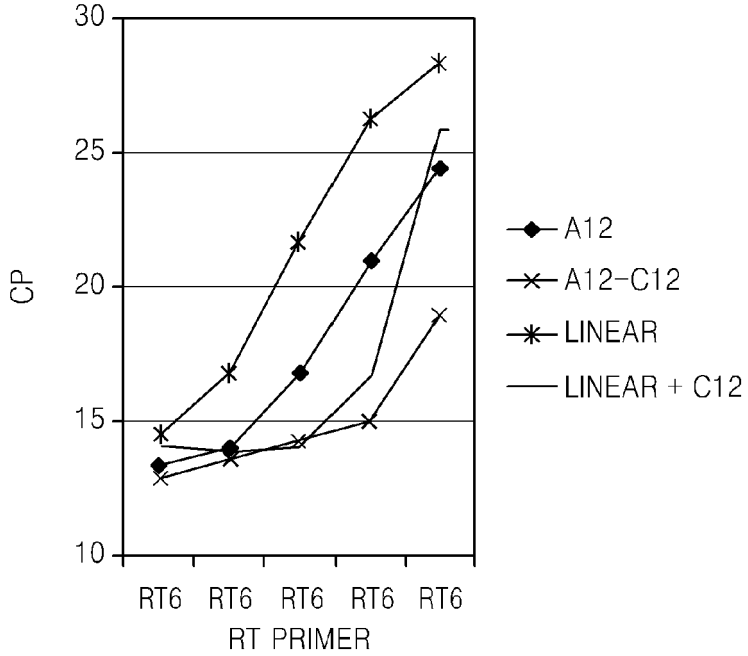

30
25
20
15
10
CP
RT6 RT6 RT6 RT6 RT6
RT PRIMER
A12
A12-C12
LINEAR
LINEAR + C12

FIG. 9B

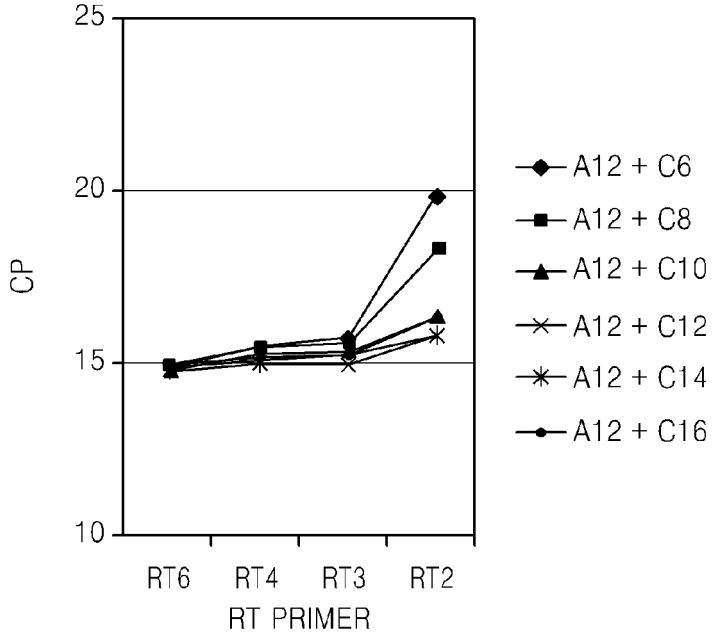
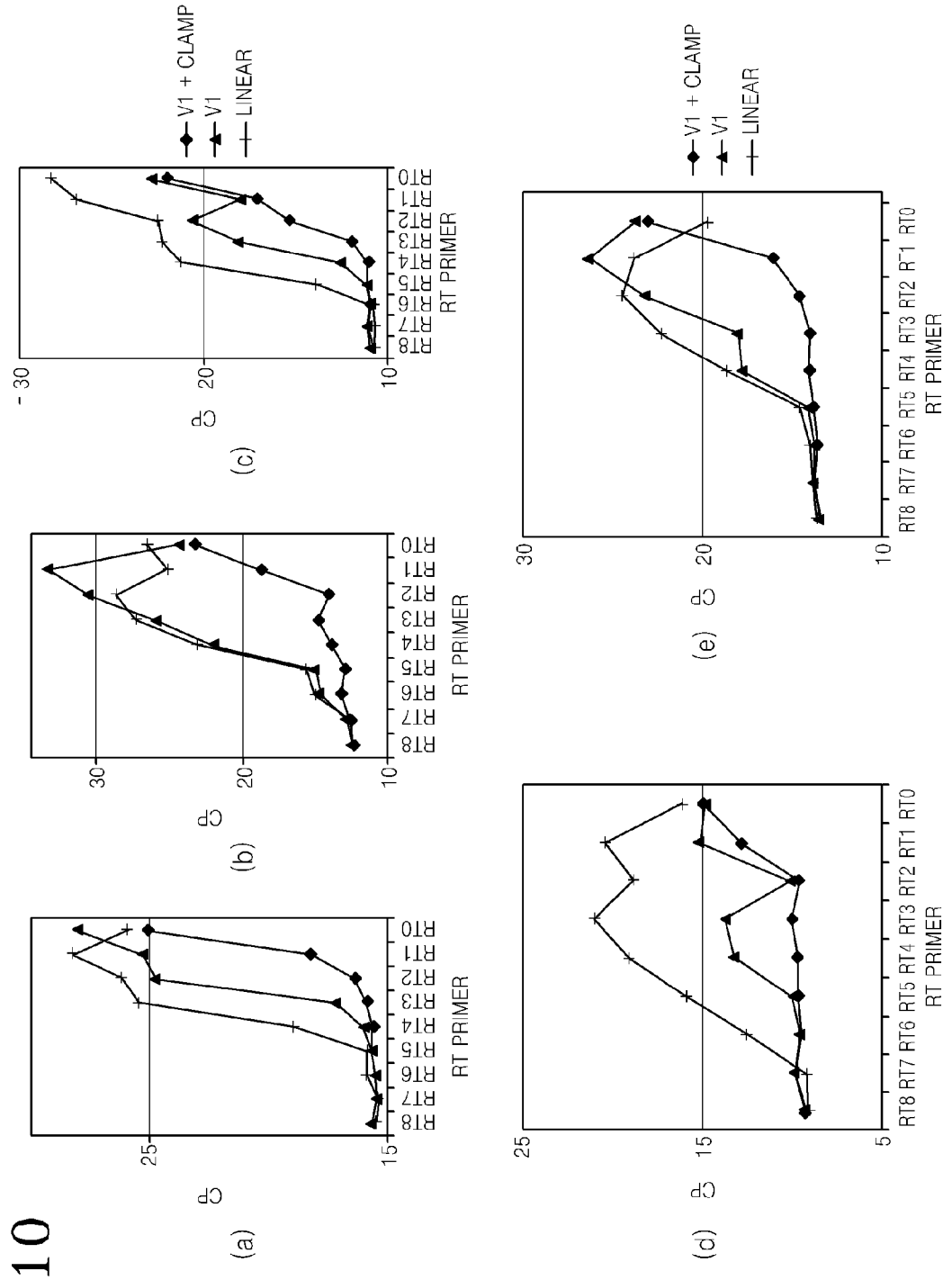

25
20
15
10
CP
RT6 RT4 RT3 RT2
RT PRIMER
A12 + C6
A12 + C8
A12 + C10
A12 + C12
A12 + C14
A12 + C16

FIG. 10


(a)
(b)
(c)
(d)
(e)
Cp
RT PRIMER
RT8 RT7 RT6 RT5 RT4 RT3 RT2 RT1 RT0
V1 + CLAMP
V1
LINEAR

Europäisches Patentamt
European Patent Office
Office européen des brevets

EUROPEAN SEARCH REPORT

Application Number
EP 13 16 4440

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (IPC)
X	DAHL F ET AL: "Circle-to-circle amplification for precise and sensitive DNA analysis", PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, NATIONAL ACADEMY OF SCIENCES, US, vol. 101, no. 13, 30 March 2004 (2004-03-30), pages 4548-4553, XP002399044, ISSN: 0027-8424, DOI: 10.1073/PNAS.0400834101 * the whole document * * figure 1; table 1 *	1-15	INV. C12Q1/68
X	US 2004/171047 A1 (DAHL GARY A [US] ET AL) 2 September 2004 (2004-09-02) * paragraphs [0045], [0293] - [0295], [0479], [0554] *	1-15	
X	WO 2010/120803 A2 (SOMAGENICS INC [US]; KAZAKOV SERGEI A [US]; KUMAR PAVAN [US]; JOHNSTON) 21 October 2010 (2010-10-21) * claims 1-10 * * figures 1-2 * * page 13, paragraph 4 - page 14, paragraph 1 * * page 15, paragraph 3 * * page 37, paragraph 4 - page 41, paragraph 2 *	1-15	TECHNICAL FIELDS SEARCHED (IPC) C12Q
X	US 6 287 825 B1 (WEISSMAN SHERMAN [US] ET AL) 11 September 2001 (2001-09-11) * claims 12,16-20 * * column 6, paragraph 2 - paragraph 3 *	1-11	

-/--

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
The Hague	28 June 2013	Bruma, Anja

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EUROPEAN SEARCH REPORT

Application Number
EP 13 16 4440

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (IPC)
X	WO 2006/108423 A2 (UNIV AARHUS [DK]; KOCH JOERN [DK]; STOUGAARD MAGNUS [DK]; LOHMANN JAKO) 19 October 2006 (2006-10-19) * figure 5 * * paragraphs [0139], [0291] * * claim 1 * -----	1-6	
X	US 2006/223098 A1 (LANE DAVID J [US] ET AL) 5 October 2006 (2006-10-05) * example 1; sequences 1,4,5 * -----	1-6	
			TECHNICAL FIELDS SEARCHED (IPC)

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
The Hague	28 June 2013	Bruma, Anja

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

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28-06-2013

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		US 2010028953 A1	04-02-2010
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		US 2006223098 A1	05-10-2006
		WO 2006105285 A1	05-10-2006

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